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Increased expression and secretion of interleukin-6 in human parvovirus B19 non-structural protein (NS1) transfected COS-7 epithelial cells

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Summary

Human parvovirus B19 (B19) has been associated with a variety of autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). We have demonstrated previously that B19 non-structural protein (NS1) induced apoptosis through the mitochondria cell death pathway in COS-7 epithelial cells and that B19 NS1 may play a role in the pathogenesis of autoimmune diseases. In order to examine the expression profiles of cytokines and chemokines in B19 NS1 transfected COS-7 cells, we constructed the NS1 gene in the pEGFP-C1 vector named enhanced green fluorescence protein gene (EGFP)-NS1. COS-7 cells were transfected with EGFP or EGFP-NS1 plasmid. The expression profiles of cytokines and chemokines, including interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, granulocyte–macrophage colony-stimulating factor (GM-CSF), growth-related oncogene α (GROα), interferon gamma-inducible protein (IP)-10, stromal cell derived factor (SDF)-1, macrophage inflammatory protein (MIP)-1β, monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T cell expressed and secreted (RANTES), Fractalkine, CX3CR1, CCR2, CCR5 and CCR11 were examined in COS-7 cells, EGFP and EGFP-NS1 transfected cells using enzyme-linked immunosorbent assay (ELISA) or reverse transcription–polymerase chain reaction (RT–PCR). Increased expression and levels of IL-6 were found in EGFP-NS1 transfected cells using RT–PCR and ELISA. There were no significant increases in the expression of IL-1β, IL-8, IP-10, SDF-1, RANTES, Fractalkine, CX3CR-1, CCR2, CCR5, CCR11, TNF-α, GM-CSF and TGF-β using RT–PCR. There were no significantly increased levels of IL-5, IL-10, TNF-α, TGF-β, GROα, MIP-1β and MCP-1 found by ELISA in this study. Our results show that increased expression and secretion of IL-6 in B19 NS1 transfected epithelial cells may play a role in the pathogenesis of autoimmune diseases.

Keywords: chemokines, cytokines, human parvovirus B19, interleukin-6, non-structural protein (NS1)

Introduction

Human parvovirus B19 (B19) infection has been associated with autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome, polymyositis and vasculitis [1–3]. In addition, B19 infection is linked to elevated levels of anti-nuclear antibody (ANA), anti-double-stranded DNA antibody, anti-neutrophil cytoplasmic antibodies (ANCA) and anti-cardiolipin antibodies (aCL) [4,5]. Recently, chronic immune activation and cytokine dysregulation have been found in patients with B19 infection [6–10]. However, the molecular basis and pathogenesis of B19-induced autoimmune phenomena is unclear. The cytotoxicity of NS1 to erythroid cells has been reported and is related to the pathogenesis of B19 infection [11,12]. NS1 has been shown to be involved in DNA replication, cell cycle arrest and the initiation of apoptosis in erythroid lineage cells [13]. The NS1 protein has also been reported to function as a transactivator of the B19 viral p6 and various cellular promoters, including

those for the expression of tumour necrosis factor (TNF)-α and interleukin (IL)-6 [14,15]. These data indicate that NS1 may play an important role in B19-induced diseases.

Cytokines regulate the immune system and may be divided into two subgroups: T helper type 1 (Th1) cytokines, mainly IL-2, IL-12, interferon (IFN)-γ and TNF-α, which activate mainly cellular immunity; and Th2 (IL-4, IL-5, IL-6 and IL-13) cytokines, which activate mainly humoral immunity [16]. Multiple cytokines are involved in the pathogenesis of autoimmune diseases such as RA and SLE [17,18]. In patients with SLE, TNF-α, IL-1, IL-4, IL-6, IL-10 and IL-18 are up-regulated [18,19]. IL-6 is classified as a multiple potent cytokine that plays an important role in inducing terminal differentiation of B lymphocytes into plasma cells and T lymphocytes into effector cells that may contribute to the production of auto-antibodies [20]. Recently, elevated IL-6, IFN-γ and TNF-α levels have been found in patients with symptomatic B19 infection [6–8]. Bluth *et al.* [9] found that Th2 cytokine responses predominated early in B19 infection, whereas late in B19 infection both Th1 and Th2 cytokine responses were elevated. Kerr *et al.* [10] found that patients with acute B19-associated arthritis have lower levels of IL-6, TNF-α and granulocyte–macrophage colony-stimulating factor (GM-CSF) than patients without arthritis. However, the role of cytokines in B19 infection is still unclear.

Because B19 replication was low in primary cell culture *in vitro* [21], we constructed and transfected the NS1 gene into epithelial cells, COS-7, for the present study. Previously, we have demonstrated that B19 NS1 induced apoptosis through the mitochondria cell death pathway in COS-7 epithelial cells [22]. In this study, we show that expression and secretion of IL-6 increase in B19 NS1 transfected epithelial cells.

Materials and methods

Plasmids

Plasmid pEGFP-C1 was obtained from Clontech Laboratories (Palo Alto, CA, USA). This plasmid contains the enhanced green fluorescence protein gene (EGFP) variant and neomycin resistant genes under the control of the cytomegalovirus early gene promoter and the SV40 early gene promoter, respectively. Plasmid pQE40-NS1, containing the NS1 gene of B19, was kindly provided by Professor Susanne Modrow, from the Institute for Medical Microbiology, Universität Regensburg, Regensburg, Germany. The NS1 open reading frame (ORF) was obtained by polymerase chain reaction (PCR) using a 5′ primer (5′-ATGGAGC TATTTAGAGGG-3′) and -3′ primer (5′-AAGTAGCA GAAATACAGGT-3′) for pQE40-NS1. The primers used for PCR contain *Bgl II* and *Sal I* recognition sequences for subsequent cloning. Amplified DNA was ligated into the *Bgl II* and *Sal I* cloning site of the pEGFP-C1 vector (EGFP). The PCR was performed with reagents containing a 0·2 μM primers mixture, 1·25 μM dNTP mixture, 1·5 μM $MgCl_2$, 10 ng template and 2·5 U DNA polymerase (Takara, Tokyo, Japan). The NS1 was fused to the COOH-terminal end of EGFP. The ligatant, EGFP-NS1, was then transformed into *Escherichia coli* DH5α competent cells, which were obtained from Life Technologies (Carlsbad, CA, USA). One set of primers was selected to amplify and confirm NS1 from the constructs. Primer A is 5′-ATGGAGCTATTTAGAGGG-3′ (sense nucleotides 436–453) and primer B is 5′-AAGTAG CAGAAATACAGGT-3′ (anti-sense nucleotides 940–922) which were amplified at a 505-base pair (bp) fragment corresponding to the NS1 coding sequence. Restriction enzyme digestion, polymerase chain reaction (PCR) and DNA sequencing analysis were used to verify the plasmid.

Cell culture and transfection

COS-7 cells were obtained originally from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator. A total of 1×10^6 cells were grown to 70% confluence in 100 mm^2 culture plates before transfection. The transfection reaction was performed by using Lipofectamine plus reagents (Invitrogen, CA, USA) with 2 μg of each plasmid, EGFP or the EGFP-NS1 constructant, according to the manufacturer's instructions. The cells were then cultured in serum-free DMEM for 12 h at 37°C in a 5% CO_2 incubator and subsequently in DMEM with 10% FBS.

Semi-quantification reverse transcription–polymerase chain reaction (RT–PCR)

All studies were carried out in a designated PCR-clean area. RNA was extracted from infected cells using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was isolated from the COS-7 cells, EGFP and EGFP-NS1 expression cells at 12–72 h. RNA samples were resuspended in diethyl pyrocarbonate (DEPC)-treated water, quantified, and then stored at –80°C until used. RNA concentration and purity were determined by a spectrophotometer by calculating the ratio of optical density at wavelengths of 260 and 280 nm. The first-strand of cDNA for RT–PCR was synthesized from the total RNA (2 μg) using the Promega RT–PCR system (Promega, Madison, Wisconsin, USA). Specific primer sets are listed in Table 1. The amplification was performed in a 50 μl reaction volume containing 1 × reaction buffer (Promega), 1·5 μM of $MgCl_2$, 200 μM of dNTPs, 1 μM of each primer and 2·5 units of *Taq* DNA polymerase (Promega, Madison, WI, USA) using a Perkin-Elmer Gene Amp PCR system 2400. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 45 s and amplification at 72°C for 45 s. The RT–PCR-derived DNA fragments, obtained by 30 PCR cycles, were subjected to electrophoresis in a 1·7% agarose gel.

Table 1. Human chemokine and cytokine cDNA primer sequences.

Gene	Primer	Length (base pairs)
IL-8		
Forward	5′-CCTGATTTCTGCAGCTCTGTGTGAAG-3′	379
Reverse	5′-ATTGCATCTGGCAACCCTAC-3′	
IP-10		
Forward	5′-CCACGTGTTGAGATCATTGCTAC-3′	383
Reverse	5′-AC ATAGCACCTCAGTAGAGCTTAC-3′	
SDF-1		
Forward	5′-CAGTCAACCTGGGCAAAGCC-3′	302
Reverse	5′-AGCTTTGGTCCTGAGAGTCC-3′	
RANTES		
Forward	5′-CGGGATCCATGAAGGTCTCCGCGGCA-3′	297
Reverse	5′-CGGAATTCCTAGCTCATCTCCAAAGA-3′	
Fractalkine		
Forward	5′-AGCCACAGGCGAAAGCAGTAGCCTGG-3′	340
Reverse	5′-TTCAGACGGAGCATTCTCCTCTGGG-3′	
CCR2		
Forward	5′-TTGTGGGCAACATGATGG-3′	887
Reverse	5′-CTGTGAATAATTTGCACATTGC-3′	
CCR5		
Forward	5′-AGAGCTGAGACATCCGTTCC-3′	505
Reverse	5′-TGATCACACTTGTCACCACC-3′	
CCR11		
Forward	5′-TCCTCCCTGTATTCCTCACAATAG-3′	310
Reverse	5′-CTGGGGACTTTAGTTACTGCCAC-3′	
CX3CR1		
Forward	5′-CCGTGCTCCGCAATGTGGAA-3′	455
Reverse	5′-GCTCAGAACACTTCCATGCC-3′	
IL-1β		
Forward	5′-ATGGCAGAAGTACCTAAGCTCGC-3′	802
Reverse	5′-ACACAAATTGCATGGTGAAGTCAGTT-3′	
IL-6		
Forward	5′-ATGAACTCCTTCTCCACAAGCGC-3′	628
Reverse	5′-GAAGAGCCCTCAGGCTGGACTG-3′	
TNF-α		
Forward	5′-TTCTGTCTACTGAACTTCGGGGT-3′	184
Reverse	5′-GTATGAGATAGCAAATCGGCTGACGG-3′	
GM-CSF		
Forward	5′-TGCAGAGCCTGCTGCTCTTG-3′	400
Reverse	5′-CAAGCAGAAAGTCCTTCAGG-3	
B19-NS1		
Forward	5′-ATGGAGCTATTTAGAGGG-3′	505
Reverse	5′-AAGTAGCAGAAATACAGGT-3′	
GAPDH		
Forward	5′-CATGTTCGTCATGGGTGTGA-3′	301
Reverse	5′-AGTGAGCTTCCCGTTCAGCTC-3′	

IL: interleukin; IP: interferon gamma-inducible protein; SDF: stromal cell derived factor; RANTES: regulated upon activation normal T cell expressed and secreted; TNF: tumour necrosis factor; GM-CSF: granulocyte–macrophage colony-stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Following staining with ethidium bromide, the gels were photographed and band intensity was measured under UV light using the Alphaimager 2200 (Alphalnnotech, San Leandro, CA, USA). The specific RNA level of every sample was expressed as the product's intensity. cDNA encoding glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was amplified and quantified for each sample.

Cytokine enzyme-linked immunosorbent assay (ELISA)

The quantification of cytokine levels from the cell culture mediums was performed by duplication using ELISA kits for IL-5, IL-6, IL-10, transforming growth factor (TGF)-β, TNF-α, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 β (Biosource

International, Inc., CA, USA) and growth-related oncogene α (GROα)/melanoma growth stimulating activity (MGSA) (IBL Co. Ltd, Gunma, Japan), according to the manufacturer's instructions. In each case, the optical density of known standards was used to construct a calibration curve and the mean cytokine values ± s.d. were then calculated for each sample.

Statistical analysis

Statistical analysis was performed using the paired *t*-test. A *P*-value < 0·05 was considered significant.

Results

Expression of IL-6 in B19 NS1 transfected COS-7 cells by semiquantification RT–PCR

Total RNA from COS-7 cells (a), EGFP (b) and EGFP-NS1 (c) transfected cells were collected at 12, 24, 48 and 72 h. As shown in Fig. 1, increased expression of IL-6 was found in EGFP-NS1 transfected cells (Fig. 1c) compared to GAPDH in COS-7 cells (Fig. 1b) and EGFP transfected cells (Fig. 1b) by semiquantification RT–PCR at 24, 48 and 72 h. In additional, there were no significant increases in the expression of IL-1β, IL-8, IP-10, stromal cell derived factor (SDF)-1, regulated upon activation normal T cell expressed and secreted (RANTES), Fractalkine, CX3CR-1, CCR2, CCR5, CCR11, TNF-α, GM-CSF and TGF-β found by RT–PCR. NS1 was noted after 24 h in Fig. 1c.

The secretion of IL-6 in B19 NS1 transfected COS-7 cells by ELISA

As shown in Table 2, the amount of MIP-1β was nearly consistent in the culture mediums of COS-7, EGFP and EGFP-NS1 transfected COS-7 cells and the values were 24·1 ± 3·7 (pg/ml), 30·6 ± 5·6 (pg/ml) and 29·4 ± 7·8 (pg/ml), respectively. The values of IL-6 concentration in the culture mediums of COS-7 cells, EGFP and EGFP-NS1 transfected COS-7 cells were 190·2 ± 17·2 (pg/ml), 192·1 ± 12·3 (pg/ml) and 434·6 ± 3·15 (pg/ml), respectively. The amount of IL-6 increased in the culture medium of EGFP-NS1 transfected

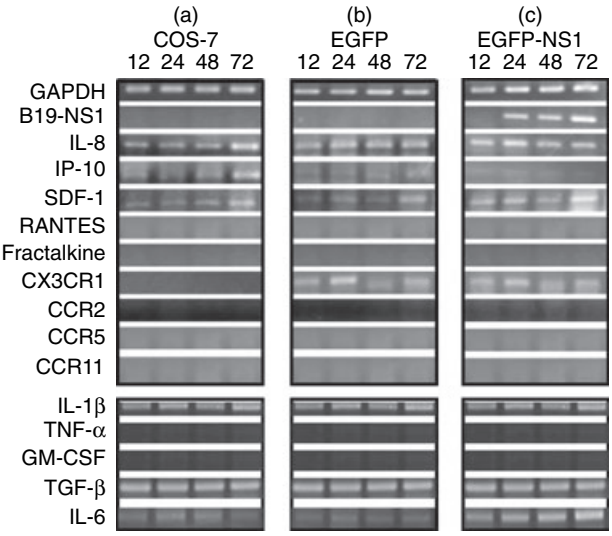

Fig. 1. Expression of various cytokines and chemokines in B19 NS1 transfected COS-7 cells using reverse transcription–polymerase chain reaction (RT–PCR). Total RNA of COS-7 cells (a) and cells transfected with enhanced green fluorescence protein gene (EGFP) (b) and EGFP-NS1 (c) were harvested at 12, 24, 48 and 72 h. RT–PCR was performed to detect the expression of various cytokines and chemokines including interleukin (IL)-1β, IL-6, IL-8, transforming growth factor (TGF)-β, granulocyte–macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, interferon gamma-inducible protein (IP)-10, stromal cell derived factor (SDF)-1, macrophage inflammatory protein (MIP)-1β, regulated uupon activation normal T cell expressed and secreted (RANTES), Fractalkine, CX3CR1, CCR2, CCR5 and CCR11. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was the control. COS-7 cells were used as the background. There were marked increases of IL-6 in EGFP-NS1 transfected COS-7 cells (c) after 24 h. NS1 was noted after 24 h in EGFP-NS1 (c).

Table 2. Levels of cytokines and chemokines in culture supernatants of COS-7, EGFP and EGFP-NS1 transfected cells.

	COS-7 cells	EGFP-transfected cells	EGFP-NS1 transfected cells
Cytokines			
IL-5 (pg/ml)	n.d.	n.d.	n.d.
IL-6 (pg/ml)	190·2 ± 17·2	192·1 ± 12·3	434·6 ± 31·5
IL-10 (pg/ml)	n.d.	n.d.	n.d.
TNF-α (pg/ml)	n.d.	n.d.	n.d.
TGF-β (pg/ml)	n.d.	n.d.	n.d.
Chemokines			
GRO-α (pg/ml)	n.d.	n.d.	n.d.
MIP-1β (pg/ml)	24·1 ± 3·7	30·6 ± 5·6	29·4 ± 7·8
MCP-1 (pg/ml)	n.d.	n.d.	n.d.

n.d.: Non-detectable; IL: interleukin; TNF-α: tumour necrosis factor-alpha; TGF-β: transforming growth factor-beta; GROα: growth-related oncogene α; MIP-1β: macrophage inflammatory protein-1; MCP-1: macrophage chemoattractant protein-1; EGFP: enhanced green fluorescence protein. gene.

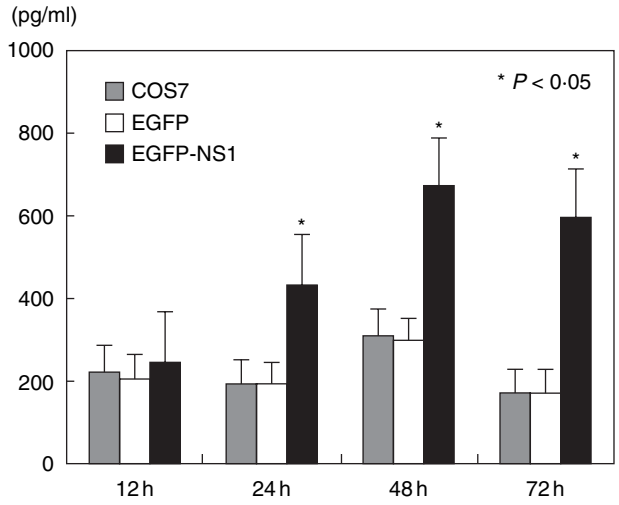

Fig. 2. Increased secretion of interleukin (IL)-6 in B19 NS1 transfected cells. COS-7 cells were transfected with enhanced green fluorescence protein gene (EGFP) or EGFP-NS1 plasmid. The supernatants were collected from cell cultures at 12, 24, 48 and 72 h and enzyme-linked immunosorbent assay was performed to evaluate the amount of secreted IL-6. The supernatant from the cell culture of COS-7 cells was used as a control. The star symbol indicates a significant difference. A significant increase of IL-6 was observed in EGFP-NS1 transfected COS-7 cells after 24 h ($P < 0{\cdot}05$).

cells compared to those of the COS-7 and EGFP transfected cells. Also, there were no significant increases in the expression of IL-5, IL-10, TNF-α, TGF-β, GROα, MIP-1β and MCP-1 found by ELISA. Furthermore, we analysed the kinetics of IL-6 secretion at 12, 24, 48 and 72 h, and a significant increase of IL-6 was observed in EGFP-NS1 transfected COS-7 cells compared to the EGFP-transfected COS-7 cells and to the COS-7 cells at 24, 48 and 72 h ($P < 0{\cdot}05$) (Fig. 2). The results show that B19 NS1 could induce the expression of IL-6 in COS-7 cells.

Discussion

We have demonstrated previously that NS1-induced apoptosis in COS-7 cells occurred through the mitochondria-mediated apoptosis pathway [22]. NS1 of B19 may play some roles in the pathogenesis of B19-induced diseases. In the present study, we ascertained the expressions and levels of cytokines and chemokines in B19 NS1 transfected COS-7 epithelial cells. It was found, using RT–PCR and ELISA, that IL-6 expressions and levels increased in EGFP-NS1 transfected cells. In addition, there were no significant increases in the expression of IL-1β, IL-5, IL-8, IL-10, GROα, IP-10, SDF-1, RANTES, MCP-1, MIP-1β, Fractalkine, CX3CR1, CCR2, CCR5, CCR11, TNF-α, GM-CSF and TGF-β.

B19 infection has been associated with various forms of autoimmune diseases and may mimic SLE in children and adults [23,24]. There are striking analogies between the clinical features and haematological findings of SLE and those of B19 infection. B19 infection has also been reported to trigger or exacerbate SLE [25,26]. Cytokine profiles in patients with SLE have been studied extensively [18]. The expression of IL-2 in freshly prepared SLE peripheral blood mononuclear cells (PBMCs) increased when compared to the PBMC control. The spontaneous production of IL-10 from SLE peripheral blood B cells and monocytes was significantly higher than that of controls. Moreover, serum IL-6 and IL-10 concentrations were higher in patients with SLE than in controls. In contrast, the production of IL-12 was found to be impaired in stimulated PBMCs from patients with SLE compared with the matched controls. These phenomena are due probably to the shifting of T helper 1 (Th1) to T helper 2 (Th2) immune responses [18]. However, cytokine profiles in patients with B19 infection are still vague. In this study, we provide evidence that NS1 induces IL-6 production, but not other cytokines and chemokines, in EGFP-NS1 transfected cells. This evidence thus suggests that NS1 induced IL-6 secretion is important in the context of B19-associated arthropathy.

Elevated IL-6 levels have been found in infants with B19 infection and lymphocytic myocarditis [7]. An increased transcript mRNA level of IL-6 was also reported in a patient with acute B19 infection and polyarthritis [27]. In contrast, Kerr *et al.* [10] discovered that patients with acute B19-associated arthritis have lower levels of IL-6 than patients without arthritis. Mitchell *et al.* [28] determined that immune recognition of NS1 may be more indicative of a recent infection with, or exposure to, B19 than association with the development of arthropathy, although an immune response to NS1 occurred at a higher frequency in patients who developed acute and chronic joint manifestations. B19 NS1 proteins have also been reported to up-regulate the IL-6 transcription (but not TNF-α, IL-1β or IL-8) in primary human haematopoetic and endothelial NS1-transfected cells [13]. It has been suggested that IL-6 might be important in the pathogenesis of certain B19 virus-associated phenomena, such as autoimmunity and arthralgia. In our study, increased expression and secretion of IL-6 was observed in B19 NS1 transfected epithelial cells. It is therefore likely that a wide variety of cells, including permissive and non-permissive cells, would secrete IL-6 when NS1 of B19 was transfected into cells. Through NS1 expression, B19 may contribute to the immune dysregulation and induce B19-associated disorders. Hence, NS1 transactivation of proinflammatory cytokine promoters such as IL-6 may be pivotal in triggering the various inflammatory and autoimmune disorders that have been linked to B19 infections.

In a previous study, we have demonstrated the association of B19 infection with the production of ANCA and aCL [4]. As IL-6 can stimulate polyclonal B cell activation [20], the elevation of IL-6 production via the viral transactivator protein NS1 may contribute to the production of ANCA and aCL. Hence, further study is required to clarify whether

other cytokines, in addition to IL-6, are involved in the production of autoantibodies followed by B19 infection in epithelial cells. In conclusion, our results provide a clue that B19 NS1 may play a role in cytokine modulation and the pathogenesis of autoimmune diseases.

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